CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

Department of Crop Sciences and Agroforestry

Use of *in vitro* cultures in *Grevillea banksii*

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Supervisor:

Ing. Iva Viehmannová, Ph.D.

Author:

Karolína Pumprová

Consultant:

Ing. Jan Vítámvás, Ph.D.

Declaration

I declare, that a bachelor thesis "Use of *in vitro* cultures in *Grevillea banksii"* is original result of my work and that I have used only resources in references.

Prague, April 2016

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Karolína Pumprová

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Abstract

Grevillea banksii (Proteaceae) is original Australian plant with very attractive flowers and a great economic potential as a cut flower on the global ornamental plant trade. The aim of this thesis was to optimize suitable protocol for rapid and effective *in vitro* micropropagation of this species. A propagation protocol was developed for establishment of *in vitro* culture, and multiplication and rooting of plants. Establishment *in vitro* was successfully provided by seeds, surface sterilised with 90% EtOH and 2% NaClO. For optimization of multiplication medium full-strength Murashige and Skoog (MS), half- strength Murashige and Skoog (½ MS) and Woody Plant Medium (WPM) media with various concentrations 0-1 mg/l of N6-benzylaminopurine (BAP) were used. Indole-3-butyric acid (IBA) was tested for root induction at concentrations 0.1 and 0.5 mg/l. After eight weeks of cultivation, from all tested types of media, MS medium supplemented with 0.1 BAP mg/l provided the highest length of plant (2.97 cm), number of buds per explant (7.4) and number of shoots per explant (1.75). Formation of roots was successful using both concentrations of IBA. Explants cultured on 0.5 IBA mg/l developed higher number of roots per explant (2.4). Rooting percentage in this treatment was 80% and thus, this medium can be recommended for rooting of this species. The process of propagation optimized within this thesis may be used for mass propagation of ornamental plant *Grevillea banksii*.

Keywords: *Grevillea* · *in vitro* propagation · plant growth regulator · Proteaceae · rooting

Abstrakt

Grevillea banksii je původem australská rostlina s velmi atraktivními květy a velkým ekonomickým potenciálem jako květina k řezu. Cílem této práce bylo optimalizovat protokol pro rychlé a efektivní množení tohoto druhu v *in vitro* podmínkách. Výstupem práce bylo zavedení rostlinného materiálu *in vitro*, optimalizace médií pro množení a zakořeňování *in vitro*. Zavedení *in vitro* kultury bylo provedeno prostřednictvím povrchové sterilizace semen, pomocí 90% EtOH a 2% NaClO. Pro optimalizaci *in vitro* množení byla testována kultivační média Murashige and Skoog (MS), poloviční Murashige and Skoog (½ MS) a Woody Plant Medium (WPM) s cytokininem 6-benzylaminopurinu (BAP) o koncentracích 0-1 mg/l. 3-indolylmáselná kyselina (IBA) byla testována pro zakořeňování výhonů. Byla využita v koncentracích 0,1 a 0,5 mg/l. Po osmi týdnech kultivace vykazovalo MS médium s přídavkem 0,1 BAP mg/l nejlepší výsledky u měřených charakteristik rostlin: výška rostliny (2,97 cm), počet pupenů na rostlinu (7,4) a počet nových výhonů na rostlinu (1,75). Zakořeňování bylo úspěšné v obou uvedených koncentracích IBA, nicméně varianta 0,5 mg/l IBA byla průkazně úspěšnější v počtu kořenů na rostlinu (2,4) a rovněž procento zakořeňování bylo vyšší (80%). Proces optimalizovaný v této práci může být použit pro komerční množení okrasného druhu *Grevillea banksii*.

Klíčová slova: *Grevillea* · *in vitro* množení · Proteaceae · růstové regulátory · zakořeňování

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Abbreviation list

ACRA	Australian Cultivar Register Authority
AND	Andersen medium (Anderson, 1975)
ANPS	Australian Native Plant Society
BAP	6-benzylaminopurine
DNA	Deoxyribonucleic acid
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KIN	Kinetin
MS	Murashige and Skoog (1962) medium
NAA	α – naphthaleneacetic acid
NSW	New South Wales
PCR	Polymerase Chain Reaction
RAPD	Random amplified polymorphic DNA
PGR	Plant growth regulator
PVP	Polyvinylpyrrolidone
WPM	Woody Plant Medium (Lloyd and McCown, 1981)
ZEA	Zeatin

1 Introduction

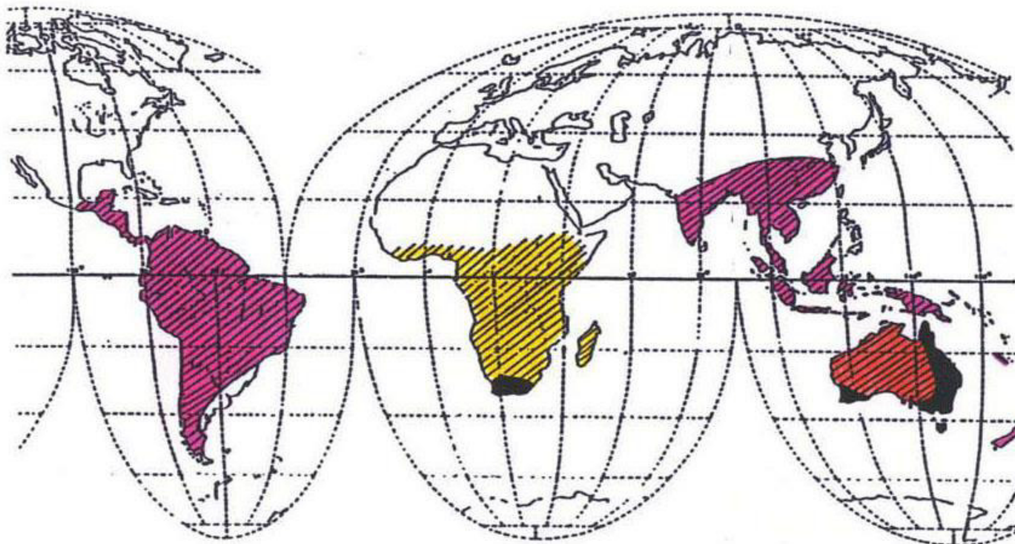
Grevillea banksii, commonly known as an ornamental garden plant, is a species belonging to Proteaceae family. Origin of the species is in Central eastern Queensland (Wilson, 2000). *Grevillea* and her hybrids or cultivars has a great potential as ornamental flowers (Martin and Brown, 2007). *Grevillea* is one of four genera and/or species which were identified from the Top End region potentially capable to meet export market criteria set by Rural Industries Research and Development of Corporation of Australia (Marcsik et al., 2006). *Grevillea* are generally considered as difficult-to-root species (Dupee and Clemens, 1982). Under natural conditions, seeds remain dormant and germination can begin after coat is damaged by fire or abrasion (Stevens, 1978). Propagation by seeds, cuttings and grafting in many species is not very successful and it is often a long term matter (Morris, 2002; ANPS, 2009; Webb, 2013). Micropropagation provides opportunity to produce high range of genetically uniform explants in short time and at competitive prize (Jha, 2005, Deberg and Zimmerman, 2012). There have been reported some protocols for *Grevillea* species propagation. Watad et al. (1902) used *in vitro* techniques for six species and cultivars of *Grevillea*: *G. pinaster*, *G. crithmifolia*, *G. petrophiloides*, *G.* 'Roundo', *G.* 'Robyn' Gordon' and *G.* 'Robyn Hood'. This research showed a positive effect of BAP on shoot production, same as Leonardi et al. (2001) observed for *Grevillea rosmarinifolia* and *Grevillea x semperflores*. IBA and NAA were also tested in these two treatments. Effective protocol for mass production of *Grevillea banksii* have not been optimized yet. The main objective of this research was to develop suitable protocol for establishment of *in vitro* culture, propagation of plants and *in vitro* rooting of shoots. Effect of basal medium types MS (Murashige and Skoog, 1962) at full and half-strength and WPM (Lloyd and McCown, 1980) in combination with BAP and IBA at various concentrations were tested.

2 Literature review

2.1 Taxonomy

According to Angiosperm Phylogeny Group (2009) Proteales order includes 4 families Nelumbonaceae, Platanaceae, Proteaceae and Sabiaceae. Nelumbonaceae consist of only one aquatic genus *Nelumbo* with two species (Watson and Dallwitz, 1992), *Nelumbo lutea* known as American lotus and *Nelumbo nucifera* known as sacred or Indian lotus (eFloras, 2008). Platanaceae has also one genus *Platanus* with 10 species native to Northern hemisphere. Sabiaceae with one genus *Sabia* and 55 species (Watson and Dallwitz, 1992) has unresolved systematic position, but most recent chloroplast genome analysis of Xue et al. (2012) confirmed close relationship with their sister taxas Platanaceae and Nelubonaceae.

Proteaceae family contents 79 genera divided into 7 subfamilies, 12 tribes and comprise about 1700 species. In Australia 49 genera occur with approximately 1100 species, of which 37 genera are endemic (Orchard, 1995). In South Africa occur 14 genera with 400 species mostly from subfamily Proteoideae (Fig 1.). *Brabejum,* occurring in Western Cape is only genus which belongs to subfamily Gravilleoideae. In South America 8 genera occur containing 85 species and in East Asia 34 genera occur (Prance, 2007).



Proteoideae, Grevilleoideae, Proteoideae and Grevilleoideae, Centre of diversity

Figure 1: World distribution of Proteaceae (Composite of sources: Venkata Rao, 1971; Johnson and Briggs, 1975)

Bellendenoideae, Caranarvonioideae, Eidotheoideae, Grevilleoideae, Persoonioideae, Proteoideae, and Sphalmioidea are subfamilies of Proteaceae (Kiu, 1988). Johnson and Briggs proposed evolutionary relationship (Fig 2) of Grevilleoideae in 1963. Later was Grevilleoideae divided into seven tribes: Oriteae, Knightieae, Embothrieae, Grevilleeae, Helicieae, Macadamieae, Banksieae (Prance, 2007).

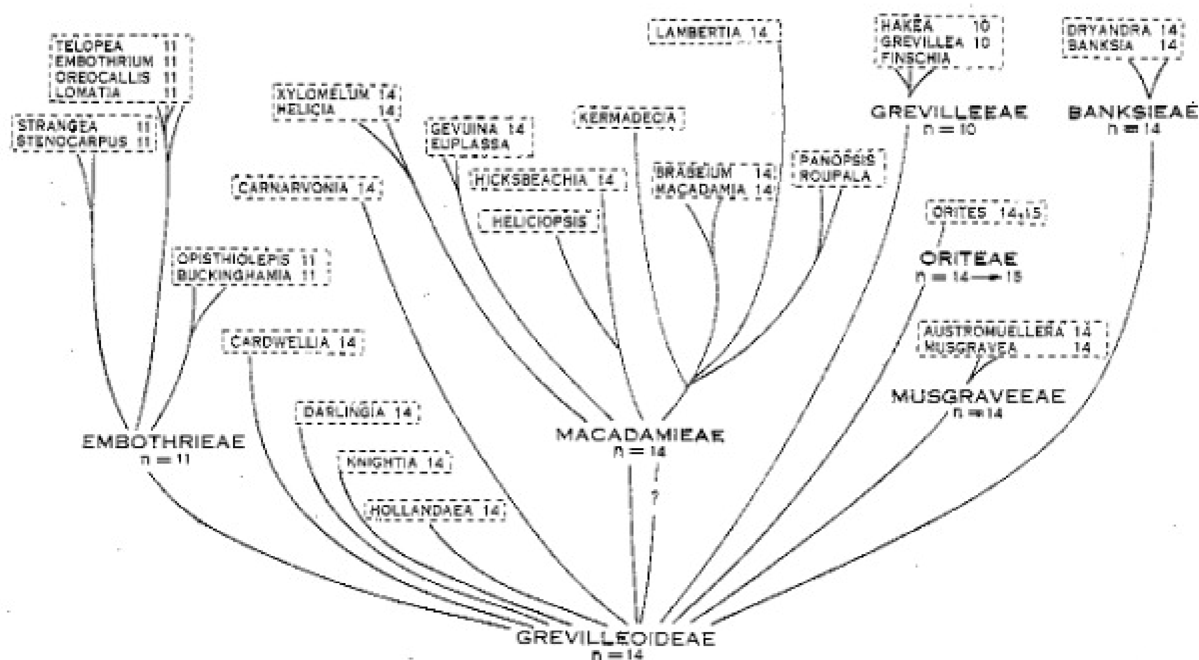


Figure 2: Suggested evolutionary relationship in Grevilleoideae (source: Johnson and Briggs, 1963)

Grevillea is with two other genera, Australian endemic *Hakea* and Malesian *Finschia* included in Grevilleeae (Johnson and Briggs, 1975). It is the third-largest genus of Australian eudicots (Wilson, 2000). Two recent treatments (McGillivray and Makinson 1993, Olde and Marriott 1994, 1995) recognise 357 Australian species of *Grevillea,* with 355 endemic. Outside of Australia, there are 3 species in New Guinea, 3 species in New Caledonia, and one endemic in Sulawesi.

2.2 Origin, distribution and history

Proteaceae evolved from gymnosperm species (Paterson-Jones, 2000) in the late Paleozoic 360 to 125 million years ago (Levin, 2016). This family is of Gondwanian origin (Vogts, 1982) and today has two centres of diversity (Fig. 1) in southern Africa

and Australia (Prance, 2007). Barker et al. (2007) apply molecular dating to Deoxyribonucleic acid (DNA) sequences from over 40 Proteaceae species. This research supported hypothesis about 'drift' of Australasian and South American ancestors of current species. Unexpectedly, there was affinity between some of South African and Australian Proteaceae which means, that some of them has to be blown across the Indian Ocean much later.

According Weston (2007), the most diverse geographical areas of Proteaceae family are in Australia, followed by southern Africa, South America, New Caledonia, New Guinea, Malesia, South and East Asia, tropical Africa, Central America, Madagascar, New Zealand, Fiji, southern India, Sri Lanka, Vanuatu and Micronesia (Paterson-Jones, 2000). South-west of Western Australia, the eastern seaboard between about East Gippsland and Marybourgh and island to the Great Diving Range, the Kimberley region of Western Australia and 'Top End' of the Northern Territory are the main Australian geographical areas where occur *Grevillea* genus. There are more than 350 species endemic to Australia including *Grevillea banksii.* The original distribution of *Grevillea banksii* is Central eastern Queensland (Wilson, 2000).

The first *Grevillea* taxon was collected by Joseph Banks and Daniel Solander on Cook's voyage in 1770. Solander's manuscript named *'Leucandendroides'* showed immediately affinities with South African Proteaceae. After settlement in 1788, some collections were forwarded from Sydney to Britain by early colonists. Robert Brown arrived in Australia in 1801, and collected a vast array of Australian plants including a lot of grevilleas. The genus name was initially established as '*Grevillia'* by Joseph Knight in 1809, honours Charles Francis Greville (1749-1809) (Makinson, 2000).

Spelling *'Grevillea'* by Robert Brown was conserved under the International Code of Botanical Nomenclature and was published in 1959. According to Wilson (2000) Robert Brown named 79 taxa in *Grevillea.* In August 1882 Brown also collected *Grevillea banksii* between Facing and Curtis Islands, near Gladstone and named after Sir Joseph Banks (Wilson, 2000).

Grevillea banksii unevenly distributed in Queensland from Yeppoon to about Ipswich, commonly near the coast but extending inland near Eidsvold and the upper Brisbane (Fig. 3). It grows in various habitats, usually in woodland or open forest, normally on flatter sites in sandy soils but sometimes in heath or on rocky hillsides (Wilson, 2000).

Figure 3: ***Grevillea banksii*** **distribution (source: Australian National Botanic Gardens, 2010)**

2.3 Morphology and anatomy

The Proteales order, dicotyledonous flowering plants, is part of peripheral eudicots in Angiosperm Phylogeny Group III (Berry, 2015). They are usually bisexual evergreen trees, shrubs and herbs (Berchtold and Presl, 1820), in the case of Sabiaceae also lianas (Watson and Dalviz, 1992). All the Proteales families are similar in having 1 ovule per ovary. Nelumbonaceae and Platanaceae have similarities in having apical placentation. Platanaceae with Proteaceae have very similar wood anatomy (Simpson, 2010); presence of five capillary bundles, tannin tissues in the carpels, usually one or two large orthotropous ovules and floral organs which may be arranged in dimerous whorls (Kubitzki, 2007).

2.3.1 Proteaceae

Proteaceae are trees or perennial (Weston, 2007) and acaulescent shrubs (Stevens, 2001). Mostly shrubs or small trees ranging from herbaceous to woody trees over 40 m tall. In majority evergreen, nerveless there is one exception in South America, *Embothrium coccineum* is facultatively deciduous. Some species are dioecious or andromonoecious but usually completely bisexual. Proteaceae have lateral roots of limited grown, forming clusters (Weston, 2007). These roots were secondary lost in

subfamilies Persoonioideae and Symphionematoiedae (Lee, 1978). Plants are rarely mycorrhizal (Stevens, 2001).

Leaves are alternate, opposite or whorled; simple or variously divided. Inflorescences are areaxillary, ramiflorous, cauliflorous, or terminal, simple or rarely compound, with flowers borne laterally either in pairs or sometimes singly, racemose, sometimes spicate, paniculate, or condensed into a head; bracts subtending flower pairs usually small, sometimes accrescent and woody; floral bracts usually minute or absent. Flowers are usually bisexual, actinomorphic or zygomorphic, hypogynous; perianth of 4 (3 in *Grevillea donaldiana* and 5 in a minority of flowers of *Bidothea hardeniana*) valvate, free or variously united tepals; stamens (3)4(5), opposite tepals, usually all fertile or sometimes 1 or more sterile; filaments are partly or wholly adnate to tepals or rarely free; anthers basifixed, usually bilocular and tetrasporangiate but occasionally the lateral anthers unilocular and bisporangiate; 1-4 hypogynous glands usually present, scale-like or fleshy, free or fused into a crescentic or annular nectary; gynoecium of 1 carpel (sometimes 2, free carpels in *Grevillea banksii*); ovary superior, sessile or stipitate, with variously positioned marginal placentae; style usually distinct, often with apex functioning as a pollen presenter; stigma small or sometimes relatively large and plate-like, terminal or subterminal; ovules 1 to many, anatropous to orthotropous, bitegmic, crassinucellate. Fruit is dehiscent or indehiscent, a follicle, achene, drupe or drupe-like. Seeds 1 to many, sometimes winged; endosperm is present or absent at maturity (Weston, 2007).

2.3.2 *Grevillea*

Representatives of *Grevillea* genus are erect to prostrate shrubs or occasionally trees, sometimes lignotuberous or root-suckering. Leaves are sessile or petiolate, entire or toothead, or divided up to three orders of division; margins flats to revolute, sometime obscure or absent; venation pinnat-reticulate to parallel. Flowers are hermaphrodite, pedicellate or sessile, usually paired, with a common bract, aggregated into conflorescences. Periath is usually zygomorfic with limb decurved, sometimes tregular with limb erect. Tepals are 4 or rarely 3 (*Grevillea donaldiana*), separating after anthesis to realese style-end. Outer and inner surfaces of periath are glabrous or hairy; ovary is superior, sessile or stipitate; ovules 2; pollen-presenter erect or oblique to lateral on style, discoid or conical. Fruit a follicle or rarely (*Grevillea candicans*) an

achene, 2- or 1- seeded, caducous or persistent, glabrous or hairy; perocarp crustaceous to bon- textured. Seeds either peripterous with usually flat-ellipsoidal seed-body, or unwinged ellipsoidal with or without terminal subtriangular waxy elaiosome, or rarely hemispherical" (Makinson, 1993).

2.3.3 *Grevillea banksii*

Grevillea banksii (Fig. 5, 6) is bushy to spindly erect shrub or slender tree, 2-10 m high, or rarely a prostrate to sprawling shrub. Leaves are 8-30 cm long, pinnatipartite (rarely the odd leaf entire); lobes 4-12 narrowly elliptic to linear, 5-18 cm long, 5-15 mm wide, not pungent; margins shortly recurved or revolute; lower surface usually mostly exposed, subsericeous to subvillous. Conflorescence is simple or few-branched; it conflorescence is erect, ± cylindrical, irregular to subsynchronous; floral rachis 50-120 (-200) mm long. Flower colour in periath is creamy white or bright scarlet to crimson, rarely pink to apricot with a yellow limb; style usually matching perianth in colour, rarely much paler. Perianth is open-tomentose to shortly pilose outside with bouth biramous and simple erect hairs. Pistil is 32-50 mm long; style sometimes with biramois hairs for up to 4 mm above ovary, otherwise glabrous, lacking a dorsal hump immediately below style-end. Follicle is 15-25 mm long, tomentose with biramous hairs mostly replaced by erect simple glandular hairs as fruit matures (Makinson, 2000).

Figure 5: *Grevillea banksii* ilustration (source: Bauer, 1813)

Figure 6: *Grevillea banksii* flowering habit (author: Plants of Hawaii, 2008)

2.4 Use

Grevillea species are mostly known as garden plants, because of high range of flower shapes and colours (Olde and Marriott, 1994). In Europe, *Grevillea* species are used for ornamental horticulture, agroforestry, and limited exploitation of timber (Makinson, 2000).

For the first time, grevilleas have been cultivated more than 200 years ago in England. Although *Grevillea* genus is native to northern part of Australia, lots of species are frost and cold tolerant to -4 °C, and suitable for cultivating in Europe (Elliot and Jones, 1990). Several species of *Grevillea* were, and are used by Aborigines for food (nectar, seeds and gums), medicines, glues, and timber for tools and weapons (Makinson, 2000). Acording to Orwa (2009), *Grevillea robusta* known as 'Silky Oak' is very important species for especially developing countries. The main products are timbre, fibre fuel, gum, resin and also honey. For example in Kenya, most of the private forests are planted by *Grevillea robusta* (Mburu, 2005). There are also essays with agroforestry systems in semi-arid areas, specifically with *Zea mays* L., for combination

of low and deep rooting species (Lott et al., 2003).

Grevillea banksii and her hybrids or cultivars has a great potential as an ornamental flower (Martin and Brown, 2007). In 2002 was the total area, planted by Proteaceous cut-flower, estimated on 10 000 hectares and the whole sale of production in Australia in 2000 reached US$ 12 million (Weston, 2007). According Gollnow (2016), Australia is part of the world flower trade. The NSW cut flower industry has an estimated farm value of A$ 202.7 million, witch 90% selling is on domestic market. Majority of exports are destined in Japan, over 50%. North Amerika takes 30% of exports. Australian native flowers and South African Proteaceae comprise 95% of exports. Native cut flower exports have increased from $3 million in 1983 to $27 million in 1995/96 (Macnish, 1999). Vase life of cut *Grevillea* is varying from three days for *Grevillea wickhamii* to nine days for *G. whiteana*. Species with long vase life as *G. pteridifolia, G. sessilis* and *G. whiteana* may by useful for breeding and cut flower production (Joyce et al., 1996). There is an effort to use these species for *Grevillea* hybrid production (Martin and Brown, 2007).

2.5 Variability in *Grevillea banksii* and plant breeding

The great variation through *Grevillea* species is in flower colour. Raymond (2013) visualised the spatial diversity in *Grevillea* flower colour across Australia. The colour starting by red (which is the most common colour), orange, yellow, white, pink, purple, and more. *Grevillea* inflorescences are mostly toothbrush-like or spider-like clusters about 5 to 12 cm length. Some have very strong aromas while the others are pleasant and sweet (Leonhardt, 1999). The attraction of flowers let to its use as cut flowers (Joyce et. al. 1996). *Grevillea banksii* has considerable variation in habit, leaf size and lobing, indumentum, and flower colour. There are two forms of wild *Grevillea banksii*. Red and white-flowered plantstady (Fig. 7) may occur together or in pure stands. Forms based on habit and distribution are distinguishable, although these do not accommodate all populations. A 'tree form' growing to 6-10 m tall is common in the area between Maryborough and Bundaberg in flat sandy forest country. The 'Townsville form', occurring between Ingham and Townsville, is generally more shrubby and has pistil length (<35 mm) and inflorescence length (usually <6 cm) at the lower end of the ranges. The 'prostrate coastal form' is sporadic along the coast over much of the range.

Usually on exposed headlands; its habit varies from truly prostrate to decumbent, with the flower heads usually raised.

The widely cultivated variant known as *Grevillea banksii* var. *forsteri* is of unknown but probably wild origin (Makinson, 2000). Possibly the first *Grevillea* hybrid was *Grevillea* 'Robyn Gordon' (Fig. 8) breaded in late 1950's by plant breeder Dave Gordon in south-western Queensland. It is a hybrid between *Grevillea banksii* and *Grevillea bipinnafida* (Downing, 2010). It exist many of hybrids and cultivars of *Grevillea banksii* and other species or hybrids (Tab. 1). The Polymerase Chain Reaction-Random Amplified Polymorphic DNA (PCR-RAP) method was used for determination parents of *Grevillea* hybrids, and shown high number of *Grevillea banksii* parentage (Pharmawati, 2013). Most of the crossings are relied and involved by *Grevillea banksii* and allied species (Martin and Brown, 2007).

Table 1: *Grevillea banksii* hybrids and cultivars (source: author based on: ACRA, 2013)

Hybrid/cultivar	Parental origin
‘Robyn Gordon’	G. banksii R.Br × G. bipinnatifida
‘Mason's hybrid’	G .banksii R.Br × G. bipinnatifida
‘Coconut Ice’	G. banksii R.Br (white form) ×G. bipinnatifida
‘Superb’	G. banksii R.Br (white form) ×G. bipinnatifida
‘Honey Gem’	G. banksii R.Br × G. pteridifolia
‘Winter Sparkles’	G. banksii R.Br × G. pteridifolia
‘Starfire’	G. banksii R.Br × G. pteridifolia
‘Misty Pink’	G. banksii R.Br × G. sessilis
‘Pink Surprise’	G. banksii R.Br × Grevillea sp.
‘Patricia Marie’	G. banksii R.Br
‘Kingaroy Slippers’	G. banksii R.Br
‘Gypsy’	G. banksii R.Br
‘Jester’	G. ‘Honey Gem’ × G. ‘Coconut Ice’
‘Sunset Splendour’	G. ‘Honey Gem’ × G. pteridiifolia
‘Starflame’	G. ‘Honey Gem’
‘Amber Passion’	G. ’Honey Gem’
‘Ladelle’	G. ‘Misty Pink’

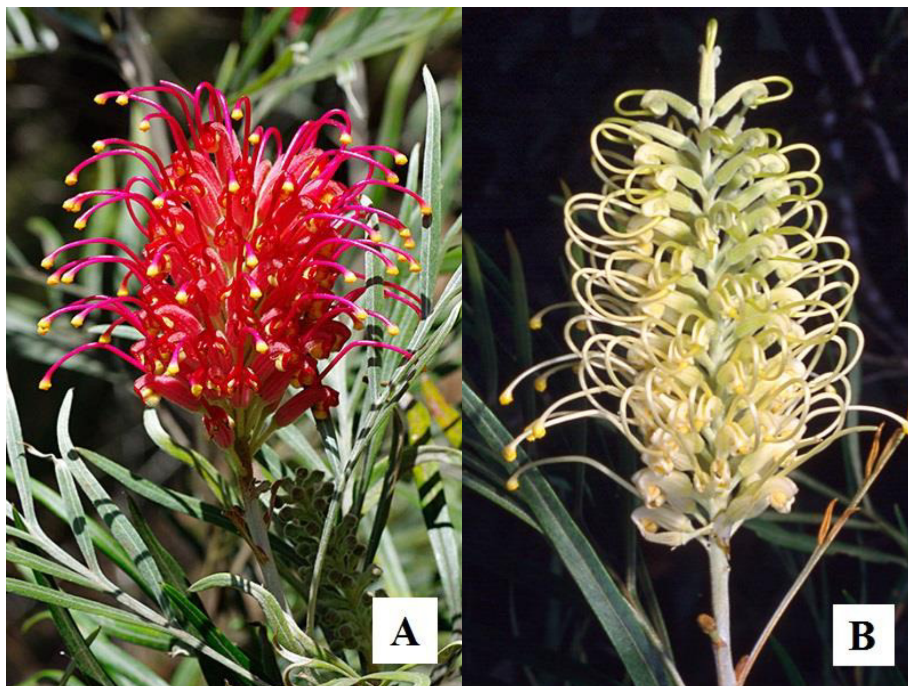


Figure 7: ***Grevillea banksii*****, A) red and B) white form (source: ANPS, 2015)**

Figure 8: ***Grevillea*** **'Robyn Gordon' (source: ANPS, 2009)**

2.6 Propagation

There are two principal methods of propagation; generative propagation by seeds and vegetative by cutting and grafting (Olde and Mariott, 1995).
Under natural conditions, seeds remain dormant and germination can begin after that this coat is damaged by fire, decay or abrasion (Stevens, 1978). Germination can be difficult. There is pre-treatment that can be used, as peeling seed coat by a sharp blade or pouring hot water over the seed (ANPS, 2009).

Cutting propagation is the most common for shrubs, indoor plants and herbaceous perennials. Stem cutting of semi-hard woods is used for Grevilleas (Mason, 2004). Generally the best time of the year for cuttings is autumn and spring, especially March, August and September on southern hemisphere. Internodal cuttings and cutting from young plant are more successful (Stevens, 1978). Most *Grevillea* species and horticultural cultivars are adapted to Australian impoverished acidic soils. In contrast, *Grevillea robusta*, the most common representative of genus, is very tolerant of the wide range of environmental conditions. In this reason is used for grafting as rootstock plant for *Grevillea*, *Azalea* or *Rhododendron* (Reid et al., 2001).

2.7 *In vitro* cultures in Proteaceae

Micropropagation is true-to-type propagation of selected genotype using *in vitro* culture techniques. Most often is micropropagation associated with mass production at a competitive price (Deberg and Zimmerman, 2012). Micropropagation has a great potential to provide high multiplication rates of selected plant genotype (Le Roux and Van Staden, 1991). Morel (1960, 1964) was the first one who described mass production of orchids. He estimated that four million plants could be achieved from one single explant of *Cymbidium*. Since Morel's prediction about *in vitro* propagation, techniques has been modified and applied on other ornamentals, fruit and nut crop, agronomic crop, medical plants and forest crop. Today, almost all of economic species has been tested on *in vitro* mass propagation (Preil, 2003). Hundreds of ornamental plants has been successfully cloned and some of them are commercially exploited e.g., chrysanthemum and carnation (Jha, 2005). This method have not been used in *Grevillea banksii* yeat, but there are studies for related species from Proteaceae family.

Considerable number of Proteaceae species and cultivars are used in

horticulture. Especially *Proteas*, *Banksias* and *Grevilleas* are widely used in cut flower industry. *In vitro* tissue cultures are used for different cut flowers, as for Proteaceae (Spencer, 2002). Multiplication of *Leucadendron* hybrids has been achieved on MS medium (Murashige and Skoog, 1962) containing 20 g/l sucrose and 3 g/l Phytogel. Multiplication rate varied between genotypes. Generally 0.025 mg/l to 1 mg/l of 6-benzylaminopurine (BAP) gave the highest number of shoots but decreased their length. 0.1 mg/l BAP gave the highest number of shoots in three hybrids tested. Culturing shoots on media containing 2 mg/l indole-3-butyric acid (IBA) resulted in 0 to 100% of shoots forming roots *in vitro* depending on genotype (Croxford et al, 2006). *In vitro* establishment and proliferation of *Leucadendron discolour* trough axillary shoot was described. A double-phase system for *in vitro* multiplication was used. For establishment phase, nodal explants were cultured on ½ MS with 3% sucrose, 0.7% agar and 0.5 mg/l BAP. Then were explants transferred on proliferation medium, this treatment did not significantly improved multiplication rate (Pérez-Francéz et al, 2001). Economically interesting and valuable for cut flower industry is *Leucadendron* 'Safari Sunset'. Multinodal explants were established on ½ MS medium supplemented with 0.5 mg/l BAP. In the establishment phase, explants cultured on a medium with 1 g/l of polyvinylpyrrolidone (PVP) developed a higher number of buds. Proliferation of axillary shoots was achieved with 0.2 mg/l BAP, being slightly higher on a medium with 0.5 mg/l BAP although the buds were deformed, showing abnormal leaves. Phytagel in this phase increased proliferation rate and shoot growth. The best rooting percentage (37.5%) was obtained with 9 mg/l IBA (Suárez et al., 2010). For *Leucadendron* 'Safari Sunset' axillary shoot proliferation was carried out on a modified MS medium containing ascorbic acid (15 mg/l) and 2% sucrose. Best results from combinations were obtained when 2.0 mg/l BAP and 2.0 mg/l gibberellic acid GA_3 were tested (Dias Ferreira et al, 2003).

Ben-Jaacov and Jaccobs (1986) reported that 10 mg/l of GA_3 and 2 mg/l BAP into Andersen medium (AND) (Anderson, 1975) was the most suitable establishment combination for *Protea cynaroides*. For *Protea obtusifolia* was used 2 mg/l GA_3 and 1 mg/l BAP as a suitable combination for multinodal shoots segment establishment (Watad et al, 1992). Multinodal shoots also used Rugge (1995) in his study with *Protea repens*. For establishment was used ½ MS medium supplemented with 1 mg/l BAP. Bud break was achieved with 6 mg/l of GA_3. For *in vitro* establishment of *Macadamia*

tetraphylla was used ½ MS medium. The highest number of shoots per plant was achieved with 2 mg/l BAP. For improvement was used GA_3 and the best combination obtained is 1 mg GA_3 + 2 mg/l BAP (Mulwa and Bhalla, 2000). Excised receptacle disks from *Telopea speciosissima* were cultured for inducing adventitious shoots on modified woody plant media (WPM) (Lloyd and McCown, 198) supplemented with BAP and IBA in various concentrations. Efficient shoot regeneration was obtained when explants were cultured on media containing 5-10 mg/l BAP and 0 - 0.2 mg/l IBA. The number of shoots produced from the nodal explants increased linearly until 0.3 mg/l BAP (Reynoso-Castillo, 2001).

Grevillea is generally considered as difficult-to-root species (Dupee and Clemens, 1982). Watad et al. (1902) used *in vitro* techniques for six species and cultivars of *Grevillea*: *G. pinaster*, *G. crithmifolia*, *G. petrophiloides*, *G.* 'Roundo', *G.* 'Robyn' Gordon' and *G.* 'Robyn Hood'. The optimal concentration of BAP was 1 mg/l. Average of new axillary shoots was 2.5- 6.2 in two months. The highest multiplication rate of 6.2 was in the case of *G. crithmifolia* and lowest 2.5 in the case of *G. petrophiloides*. *In vitro* rooting was successful with 1 mg/l α – naphthaleneacetic acid (NAA). Studying different cytokinin concentration, Leonardi et al. (2001) observed positive effects of shoot proliferation in *Grevillea rosmarinifolia* and *Grevillea* × *semperflores* up to 1-2 mg/l BAP and 5-10 mg/l kinetin (KIN). In this research were tested two auxin types. NAA give better results at 0.2 mg/l, while IBA was more effective in higher concentration. A micropropagation method for *Grevillea robusta* was developed using explants from mature trees cultured on WPM supplemented with 1 mg/l BAP and 0.05 mg/l NAA for shoot proliferation. During three transfers each nodal explant produced 3-5 shoots (Rajasekaran, 1994). For *Grevillea scapigera* was achieved micropropagation including adventitious growth from leaf section. Initiation was made with shoot tips on filter paper supports with liquid WPM supplemented with 4.64 mg/l zeatin (ZEA) and 0.7 mg/l GA_3. Thereafter the shoots were incubated on WPM solidified with agar and supplemented with 1.16 mg/l KIN and 0.11 mg/l BAP. Leaf sections of 0.7 cm^2 produced adventitious shoots after 6 to 7 weeks on ½ MS medium supplemented with 2.32 mg/l BAP and 0.11 mg/l IBA (Bunn and Dixon, 1992).

3 Aims of the thesis

The main objective of this work was development of an appropriate protocol for particular micropropagation steps of *Grevillea banksii* considered followings approaches:

- Establishment *in vitro* culture of *Grevillea banksii.*
- *In vitro* propagation and determination of suitable medium.
- *In vitro* rooting using plant growth regulators

This study was mainly focused on increasing efficiency of propagation in this species as a potential ornamental plant.

Objectives of this work were set under following hypothesis:

- 6-benzylaminopurine (BAP) is an effective PGR stimulating cell division and shoot proliferation.
- Indole- 3-butyric acid (IBA) is an auxin initiating root formation, which is necessary for *ex vitro* transfer of explants.

4 Material and methods

4.1 Plant material

Seeds were provided by Botanic garden in Bonn, Germany in 2013 via Index Seminum. Plant material was introduced *in vitro* in 2013 as describe below.

4.2 Methods

4.2.1 Establishment of *in vitro* culture

Before establishment of *in vitro* culture, the seeds (Fig. 9) were surface-disinfected using EtOH and NaClO. First of all, the seeds were rinsed with 90% EtOH for 10 seconds, thereafter they were placed on laboratory rotary shaker in a beaker with 2% NaClO solution for 20 minutes. Afterwards, in flow box, the seeds were washed with sterile distilled water three times. Then, the seeds were transferred in Erlenmeyer flaks (volume- 100 ml) containing 30 ml of ½ MS medium supplemented with 100 mg/l myo-inositol, 30 g/l sucrose, 8 g/l agar. Value of pH of the medium was adjusted to 5.7 before autoclaving. The seed coat was getting soft approximately three days after cultivation, then it was needed to peel a coat for successful germination. This was made in sterile environment of flow box on Petri dishes with scalpel, and placed back on ½ MS medium. The cultures were incubated at 25/23 °C under a 16/8 h light/dark regime, and at a photosynthetic photon flux density of 35 µmol m^{-2} s^{-1}, provided by cool-white fluorescent tubes.

Figure 9: *Grevillea banksii* seeds (source: author), Bar = 1 cm

4.2.2 *In vitro* propagation of plant material for experiment

From eight *in vitro* germinated seedlings, was randomly chosen one genotype. This seedling was cut after 6 weeks of cultivation. Cotyledonous part (Fig. 10, B) of seedling was transferred on ½ MS medium, then shoots (Fig. 10, A) were separated from mother explant and also transferred on ½ MS medium. Cotyledonous part and shoots were reutilised and multiplied. Explants were multiplied approximately every 4-5 weeks, before they started getting dry and brown on the tops or being 'glassy'. During latter cycles of multiplication were primarily used shoots (Fig. 11). Basal parts were also used for multiplication but there was inclination to form structures similar to rosettes. Explants were incubated under conditions see above.

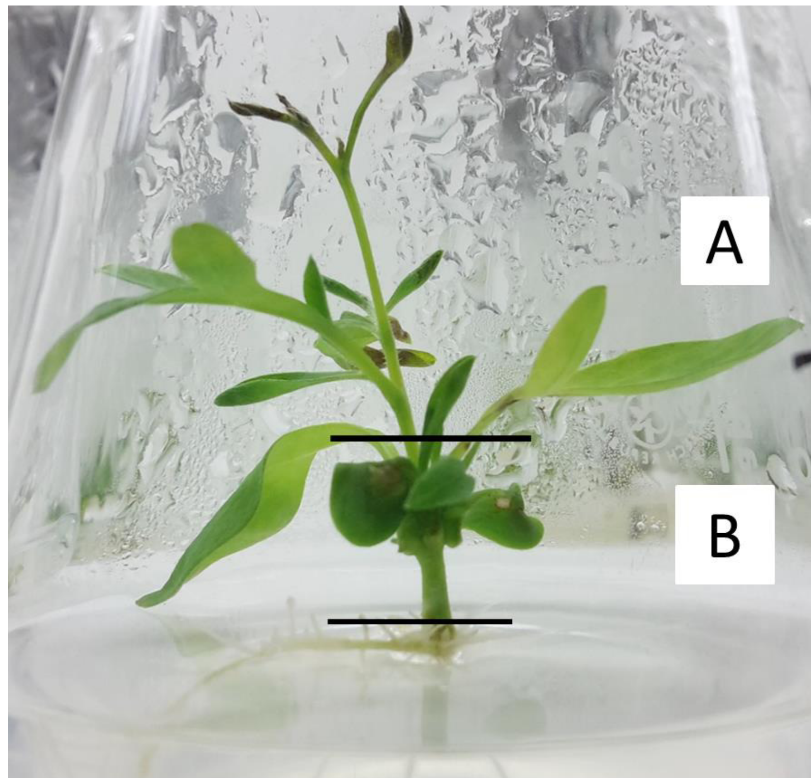


Figure 10: *Grevillea banksii* seedling: A) shoots, B) cotyledonous part (source, author)

Figure 11: Shoots used for the multiplication on ½ MS medium (source: author)

4.2.3 *In vitro* propagation experiment

For *in vitro* propagation, three basal media were tested. Full- strength **MS** medium, **½ MS** medium and **WPM,** all supplemented with 100 mg/l myo-inositol, 30g/l sucrose, 8g/l agar and with pH 5.7 adjusted before autoclaving. Each variety was supplemented with cytokinin **BAP.** Treatments are described below (Tab. 2).

Table 2: Various media and BAP combinations for propagation.

Media	BAP (mg/l)				
MS	0	0.05	0.1	0.5	1
½ MS	0	0.05	0.1	0.5	1
WPM	0	0.05	0.1	0.5	1

Cultivation was carried out under the same conditions as described above. Experiment was established from 20 explants per each treatment (media + plant growths regulator). Used explants were approximately 0.5-0.7 cm long from shoots. Propagation experiment evaluation was made after 8 weeks after cultivation. Evaluated characteristics were length of explant, number of shoots, shoot length and number of buds.

4.2.4 *In vitro* rooting

For rooting, ½ MS medium supplemented with 0.1 and 0.5 mg/l of **IBA** was used. Each treatment consisted of 20 shoots measuring 0.5- 0.7 cm. Evaluation was made after 8 weeks and measured characteristics was root length and number of roots per explant.

Statistical analysis

The statistical evaluation of morphological data was performed by analysis of variance (ANOVA), and the significantly different means were identified by using Tukey's HSD test at the 5 % level of significance ($P \leq 0.05$) (STATISTICA 12.0, StatSoft, Inc.).

5 Results

5.1 Establishement of *in vitro* culture

Standard method of seed sterilization with 90% EtOH and 2% NaClO was successful. Less than 2% of seeds in this treatment contaminated. Not scarified seeds remained inactive, but after coat remove was germination 100% successful. Germination started after 5-7 days. Seedlings were used for propagation 6 weeks after germination.

5.2 Propagation

For propagation process were tested three types of media, i.e., MS, ½ MS and WPM, media and BAP concentrations from 0-1 mg/l. In general, the most successful medium for propagation was MS medium, forming the highest plants, the highest number of buds per explant and the highest number of shoots per explant (Tab. 3). Leaves of explants cultured on MS medium were significantly larger than in two others types of media. Explants were getting hyperhydrated frequently. For this reason, it was necessary to sub-cultivate explants at least once per 4-6 weeks. Vitrification did not appeared or rarely appeared on explants cultured on ½ MS and WPM medium. In the case of ½ MS and WPM media measured characteristic decreased comparing with MS medium. WPM (Fig. 14) medium generally formed smaller explants, lower number of shoots and buds than MS and ½ MS medium. Explants cultured on WPM had the worst response. It was observed browning (Fig. 14) on basal parts of WPM explants. Some explants from variants of basal media (without BAP) formed roots spontaneously. Roots appeared around 6 week of cultivation. However, the roots were not sufficiently developed for *ex vitro* transfer.

There were apparent differences between treatments including various concentrations of BAP. Generally, growth of explant, number of shoots and number of buds decreased with higher concentration of BAP. Treatments with 0.5 and 1 mg/l of BAP formed clusters and unstable structures (Fig. 12 D, E; Fig. 13 D, E; Fig. 14 D, E). Explants were significantly smaller and often were deformed with no evidence of plant organ (Fig. 15). If appeared shoots on those treatments, they were swollen without active buds. The least satisfactory results were obtained on WPM and ½ MS media with

1 mg/l BAP forming explants around 1.1 cm high. Those combinations were not almost forming shoots and buds.

Lower concentrations of BAP influenced growth positively. The highest shoot proliferation activity was achieved in MS medium supplemented with 0.1 mg/l BAP (Fig. 12, C). Although in some characteristics, the values are not significantly higher against other treatments, for the most characteristics, this medium provided the best results: length of explant: 2.97 ± 0.27 cm, number of buds per explant: 7.40 ± 0.92 and number of shoots per explant: 1.75 ± 0.30. The second most appropriate medium for proliferation was MS with 0.05 BAP mg/l (Fig. 12, B), providing high explants: 2.11 ± 0.30 cm. From observations, explants cultured on media without plant growth regulators were healthy, with stable growth and seemed to be suitable for propagation. Shoot quality on this media was high with reduced incidence of hyperhydricity. Survival rate of explants on basal media with 0-0.1 BAP mg/l concentration was higher than for media with 0.5-1 mg/l BAP (data not shown). From this propagation protocol is evident that MS medium with low concentrations of BAP is suitable for optimal growth of explant in short time. ½ MS and WPM with no regulators could be also used for propagation.

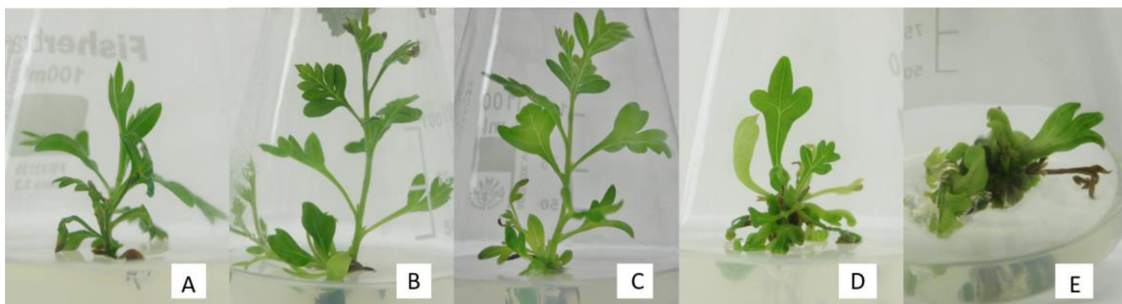


Figure 12: Explants after 8 week cultivation on MS medium supplemented, A) 0 mg/l BAP, B) 0.05 mg/l BAP, C) 0.1 mg/l BAP, D) 0.5 mg/l BAP, E) 1 mg/l BAP, (source: author)

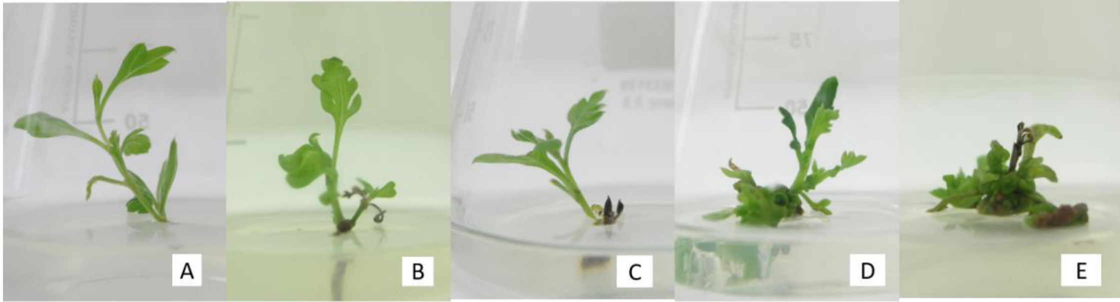


Figure 13: Explants after 8 week cultivation on ½ MS medium supplemented, A) 0 mg/l BAP, B) 0.05 mg/l BAP, C) 0.1 mg/l BAP, D) 0.5 mg/l BAP, E) 1 mg/l BAP, (source: author)

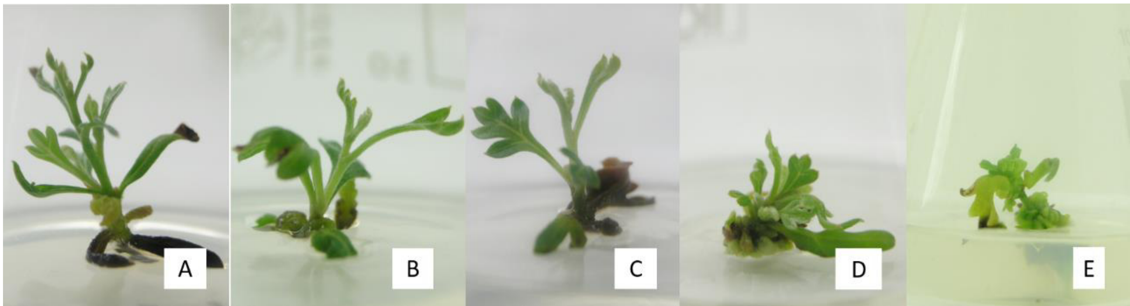


Figure 14: Explants after 8 week cultivation on WPM medium supplemented, A) 0 mg/l BAP, B) 0.05 mg/l BAP, C) 0.1 mg/l BAP, D) 0.5 mg/l BAP, E) 1 mg/l BAP, (source: author)

Figure 15: Hyperhydratation and deformation of explant with high concentration of BAP, (source: author)

Table 3: Effect of combination of basal media and BAP on various characteristics after 8 week cultivation

Basal medium	BAP (mg/l)	Length of explant (cm) (mean ± S.E)*	Number of buds per explant (mean ± S.E)*	Length of shoots (cm) (mean ± S.E)*	Number of shoots per explant) (mean ± S.E)*
MS	0	2.03 ± 0.14 bc	4.60 ± 0.88 bc	0.62 ± 0.09 ab	1.50 ± 0.32 ab
MS	0.05	2.11 ± 0.30 b	3.50 ± 0.86 ab	0.76 ± 0.18 ab	0.70 ± 0.21 bcd
MS	0.1	2.97 ± 0.27 a	7.40 ± 0.92 a	0.78 ± 0.09 ab	1.75 ± 0.30 a
MS	0.5	1.51 ± 0.12 bcde	2.45 ± 0.43 cd	0.76 ± 0.08 ab	0.84 ± 0.24 abcd
MS	1	1.54 ± 0.11 bcde	2.60 ± 0.50 cd	0.82 ± 0.05 ab	1.21 ± 0.27 abc
½ MS	0	1.76 ± 0.06 bcd	6.40 ± 0.44 cd	0.46 ± 0.05 ab	0.95 ± 0.27 abcd
½ MS	0.05	1.40 ± 0.16 cde	3.15 ± 0.72 cd	0.76 ± 0.11 ab	0.50 ± 0.15 cd
½ MS	0.1	1.38 ± 0.05 cde	2.65 ± 0.60 cd	0.62 ± 0.11 ab	0.15 ± 0.08 d
½ MS	0.5	1.08 ± 0.11 d	2.16 ± 0.47 cd	0.78 ± 0.11 ab	0.42 ± 0.15 abcd
½ MS	1	1.13 ± 0.10 d	1.19 ± 0.29 d	1.14 ± 0.21 a	0.19 ± 0.08 d
WPM	0	1.53 ± 0.07 bcde	3.45 ± 0.37 cd	0.47 ± 0.06 ab	0.20 ± 0.12 d
WPM	0.05	1.56 ± 0.06 bcde	3.25 ± 0.43 cd	0.61 ± 0.16 ab	0.30 ± 0.11 cd
WPM	0.1	1.60 ± 0.07 bcde	3.55 ± 0.21 cd	0.35 ± 0.15 ab	0.10 ± 0.06 d
WPM	0.5	1.26 ± 0.08 de	1.60 ± 0.43 d	0.80 ± 0.20 ab	0.10 ± 0.06 cd
WPM	1	1.13 ± 0.06 d	0.85 ± 0.31 d	0.75 ± 0.25 ab	0.10 ± 0.06 d

* In the same column, numbers followed by the same letter are not significantly different (Tukey's test, $p < 0.05$)

5.3 *In vitro* rooting

Explants cultured on basal media containing BAP did not formed roots for optimal *ex vitro* transfer.

Plants on medium with addition of 0.5 mg/l IBA produced significantly more roots than the plants on medium with 0.1 mg/l IBA (Table 4). 0.1 IBA formed stronger roots frequently less ramified, whereas 0.5 mg/l IBA formed thin, long roots with high number of rootlets. Explants cultured on 0.5 mg/l IBA often formed roots negatively geotropic (in the space) (Fig. 17). On the contrary, plants cultivated on medium with 0.1 IBA formed roots anchored in agar. Rooting rate was 80% in the case of 0.5 BAP. In 0.1 BAP only 40% plants rooted.

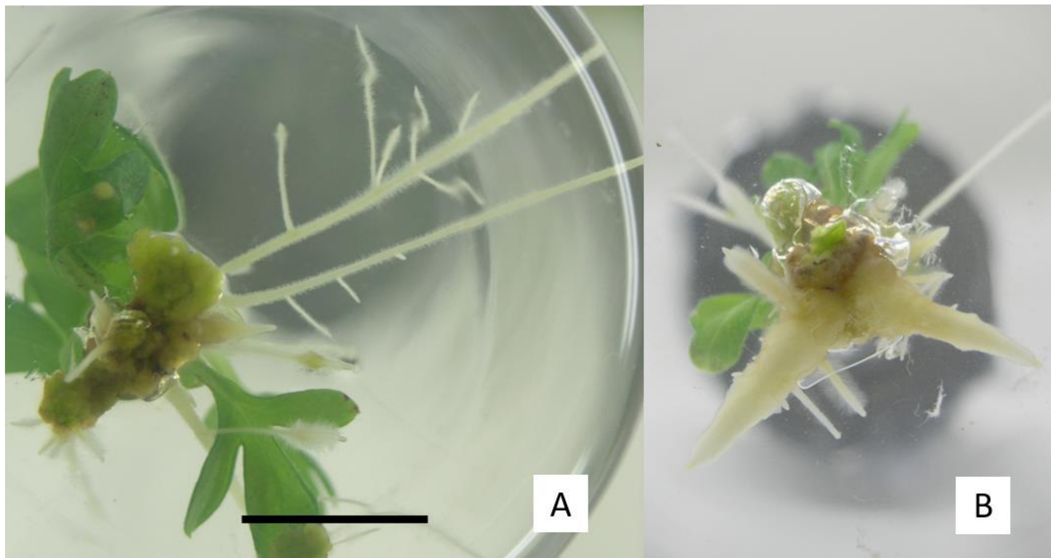


Figure 16: Plants after 8 week cultivation on ½ MS medium supplemented with A) 0.5 mg/l IBA, B) 0.5 mg/l IBA, (source, author), Bar = 1 cm

Table 4: Effect of IBA on root formation after 8 weeks of cultivation

IBA (mg/l)	Length of roots (cm)	Number of roots per explant (mean ± S.E.)
0.1	1.36 ± 0.14 ab	0.80 ± 0.26 b
0.5	1.53 ± 0.11 ab	2.45 ± 0.48 a

* In the same column, numbers followed by the same letter are not significantly different (Tukey's test, $p < 0.05$).

6 Discussion

Some micropropagation protocols has been already developed for *Grevillea* species (Watad et al., 1902; Bunn and Dixon, 1992; Rajasekaran; 1994 Leonardi et al., 2001). However, an efficient and commercially sustainable method for *Grevillea banksii* propagation had not been attempted yet.

6.1 Establishment of *in vitro* culture

NaClO is one of the most frequently chosen surface disinfection agent for seeds, applied alone, or in combination with EtOH (Sauer, 1986; Oyebanji, 2009; Sharma et al., 2012; Silva, 2015). Dixon et al. (1995) used NaClO as a seed disinfectant for two species of Western Australian plants, which has resembling characteristics as seeds of *Grevillea banksii*. Seed surface sterilisation was provided in this treatment using 90% EtOH and 2% NaClO solutions, with 98% success. The time period of NaClO application by Dixon et al. (1995) for disinfection of seeds was 3-4 min. On the contrary, in present study, seeds were submerged in NaClO solution for 20 minutes.

Germination response of species originally from south Western Australia could by related to fire response. Local Mediterranean-type climate with periodic drought and recurrent fires affect production of viable seeds (Bell et al., 1993). Morris (2002) demonstrated germination response to smoke, short heat exposure and scarification with seven *Grevillea* species. In recent study of Ma et al. (2015) was examined the effect of seed coat with germination inhibitors on seed dormancy in *Grevillea* species. Removal of seed coat dramatically increased germination. Scarification of *Grevillea banksii* ensured 100% response in germination.

6.2 *In vitro* micropropagation

In vitro propagation has a great potential to provide high multiplication rates of selected plant genotype (Le Roux and Van Staden, 1991). Most of the tissue culture studies on Proteaceae reported MS or ½ MS medium to be optimal for in vitro cultures (Croxford et al, 2006, Bunn et al., 2010; Suárez et al, 2010). The MS medium is known for its rich macro- and micro- elements, particularly nitrogen (Murashige and Skoog, 1962). Due to high concentration of elements sometimes appeared vitrifications and

browning or blackening of the explants. Leaching of phenolics may be phytotoxic and cause necrosis of the explants (Bhojwani and Razdan 1996; Krishna and Singh 2007). However, results of Croxford et al. (2006) showed very similar results as our study. Croxford et al. (2006) reported that combination of MS medium and 0.1 mg/l BAP gave the highest number of shoots in three hybrids of *Leucadendron*. With higher concentration of 0.2 mg/l BAP was successfully achieved proliferation of axillary shoots in *Leucadendron* 'Safari Sunset'. As in *Grevillea banksii* protocol, 0.5 mg/l BAP showed bud and leaf deformation (Suárez et al, 2010). On the other side, Watad et al. (1902) and Leonardi et al. (2001) observed positive effects of shoot proliferation in *Grevillea* species up to 1-2 mg/l BAP which is not in accordance with *Grevillea banksii* results.

WPM was developed for micropropagating of woody plants and contains 1/5 of nitrogen comparing to MS medium (Lloyd and McCown, 1981). However, results achieved with WPM medium in *Grevillea banksii* study were not satisfying, although Rajasekaran (1994) and Reynoso-Castillo (2001) state that WPM medium is suitable for micropropagation of two Proteaceae species. In both cases, WPM with BAP concentrations from 0.3 mg/l showed shoots proliferation. For *Protea* species was in numerous studies recommended medium containing 2.0- 10.0 mg/l GA_3 and 1-2 mg/l BAP (Ben-Jaacov and Jaccobs, 1986; Watad et al, 1992; Rugge, 1995; Dias Ferreira et al., 2003).

6.3 *In vitro* rooting

Rooting of shoots derived from *in vitro* cultures is a prerequisite to provide the physiological establishment in soil. NAA or IBA have been the most commonly used auxins for promoting rooting of *in vitro* regenerated shoots of Proteaceae. In studies of *Leucadendron*, culturing shoots on media containing 2 mg/l indole-butyric acid (IBA) resulted in 0 to 100% of shoots forming roots *in vitro* depending on genotype of (Croxford et al, 2006). Suárez et al. (2010) obtained the best rooting percentage (37.5%) with 9 mg/l IBA. Watad et al. (2012) stated that 1 mg/l NAA is suitable for *in vitro* rooting of six *Grevillea* species. In study of Leonardi et al. (2001) focused on *Grevillea rosmarinifolia* and *Grevillea* × *semperflores* were tested two auxin types, NAA and IBA. NAA give better results at 0.2 mg/l, while IBA was more effective at higher

concentration. In our study, concentration 0.5 mg/l of IBA responded in 80% of explants, whereas lower concentration (0.1 mg/l) only 40%.

7 Conclusion

The obtained results demonstrate that *Grevillea banksii* can be easily propagated and rooted *in vitro*. Establishment of *in vitro* culture was successfully achieved by surface sterilization of seeds with 90% EtOH and 2% NaClO. For *in vitro* propagation, MS medium supplemented with lower concentrations of BAP (e.g., 0.1 mg/l BAP) can be recommended. ½ MS medium supplemented with 0.5 mg/l IBA provided the highest rooting efficiency.

Therefore, micropropagation protocol optimized within this thesis might be used for mass propagation of ornamental plant *Grevillea banksii*.

Recommendation

Within following research, more types of cytokinin (either individually or in combination with auxins) for shoot proliferation, and auxins for root formation should be tested. Optimization of *ex vitro* transfer represents also very important to be solved.

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